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Suitability of Cheese Whey as a Substrate for Vitamin B12 Production by Propionibacterium Shermanii

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SUITABILITY OF CHEESE WHEY AS A

SUBSTRATE FOR VITAMIN B_{12} PRODUCTION BY

PROPIONIBACTERIUM SHERMANII

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LBB

INTRODUCTION

Disposal of wastes is a major problem confronting the food industry today. These wastes are of varied composition and origin. One such material is cheese whey. While cheese whey is an edible product, which has possible uses as human food or animal feed, the utilization of whey for these purposes has been very small when compared to the total annual production. Most of the surplus whey is considered to be and handled as an industrial waste, and disposed of in some manner.

Whey is a by-product of cheese making. In the manufacture of cheese, milk is treated with bacteria, acid or enzymes, which bring about coagulation of most of the casein of the milk. The coagulated protein along with most of the butterfat form a semi-solid material or curd which is further processed into the various cheeses. The liquid that remains after the curd has been removed, is the cheese whey.

Since 90% of the starting volume of milk remains as whey after cheese manufacture (Porges, 1959), annual production of whey is large. Webb and Whittier (1948) reported that about 10 billion pounds of whey were produced each year in the United States. In recent years approximately 12.3 billion pounds of whey are produced annually, and only 3.4 billion pounds are processed (Porges, 1959). In 1958 the United States consumption of whey was less than 25% of the total production (Wix and Woodbine, 1958). Herein lies a large part of the whey problem, the

fact that the actual consumption of whey is a small part of the total production. If consumption of whey is to increase appreciably, new uses must be found for the material. Until the consumption of whey is increased, the remaining unused volume will remain a problem for much of the industry.

Another aspect of the whey problem for the dairy industry is one of economics. Whey contains about one-half of the solids of the starting raw milk. The amount of whey disposed of each year in the United States is about 9 billion pounds (Porges, 1959). This represents 430 million pounds of sugar and 80 million pounds of protein that are discarded each year by cheese makers in the United States. From these figures it is obvious that disposal of whey as a waste is not economical from the standpoint of utilization of raw materials. Porges (1959) made an interesting observation concerning this when he stated, "I am unaware of any other industry that discards one-half of its solids in order to recover the other half."

When considering the disposal or utilization of whey, one of the first things that must be kept in mind is its chemical composition. According to Webb and Whittier (1948) a typical cheese whey contains about 6.9% total solids. Of the total solids only about 0.6% is ash or inert material and 6.3% is organic solids. The organic solids are made up of 0.3% fat, 0.9% nitrogenous compounds (calculated as protein) 4.9% lactose and 0.2% lactic acid. The lactic acid is formed from fermentation of the lactose, so the amounts of these two are somewhat

variable, but usually total 5.1%. About 55% of the nitrogenous matter of whey is heat-coagulable protein which consists of a very small portion of suspended casein, an albumin fraction and a globulin fraction.

The purpose of this study was to investigate the suitability of cheese whey as a substrate for vitamin B_{12} production by Propionibacterium shermanii. It is hoped that this work will add to the existing body of knowledge on the subject and possibly contribute to the solution of this problem.

REVIEW OF LITERATURE

Disposal. Webb and Whittier (1948) have reviewed the following methods of whey disposal:

a.) Disposal into sewers.

b.) Disposal into streams.

c.) Disposal into abandoned mine quarries or pits, or simply dumping on the ground.

d.) Disposal into prepared lagoons.

e.) Installing a sewage disposal plant to treat whey separately from municipal sewage or as a pretreatment.

f.) Returning the whey to farmers for feed.

Disposal of whey into the city sewage system is practical only when the quantity of whey is very small, or when the whey can be greatly diluted. Whey has a very high biochemical oxygen demand (BOD). The BOD of undiluted whey has been found to vary somewhat, but is generally in the range from 38,000 to 46,000 ppm compared to an average BOD for domestic sewage of about 200 ppm (Wix and Woodbine, 1958). It was also stated that when compared to domestic sewage the population equivalent to a cheese plant processing 10,000 gallons of milk per day is about 2800 persons. If whey is added to city sewage systems in anything but very small amounts, the volume plus high BOD create a heavy demand on the sewage system which may ultimately result in overloading and malfunctioning of the system. Backmeyer (1947) stated that "the problems involved in the treatment of sewage containing cheese

whey can be divided into two classes, namely (1) overload conditions resulting from 'batch' doses dumped into the sewer at infrequent intervals and (2) the continuous daily dumping of whey into the sewer systems." When 25,000 lbs. of whey was put into the sewers of a city using an activated sludge plant for sewage treatment the color of the activated sludge changed from a chocolate brown to a slate gray, the suspended solids and BOD of the effluent rose from 13 ppm to 24 to 39 ppm and the dissolved oxygen content decreased to less than 2 ppm.

Maloney et al. (1960) studied city sewage stabilization ponds containing whey wastes. In the case studied a new sewage stabilization pond did not function properly. Poor pond performance was indicated by the low concentration of dissolved oxygen and desirable algae, as well as the presence of dissolved sulfides and hydrogen sulfide odors. The malfunctioning was caused by whey wastes reaching the pond through the sewer system from a dairy plant. It was also found that these wastes had a cumulative bad effect on the pond. The addition of sodium nitrate increased the tolerance of the pond to dairy wastes, but not to any large degree and did not provide a satisfactory solution to the difficulty.

Disposal of whey by running it into streams is generally not acceptable. The large quantities of whey combined with high BOD, soon cause pollution of the stream and septic conditions. This results in the killing of fish and other aquatic life as well as noxious odors in stagnant areas (Webb and Whittier, 1948).

Dumping of whey into pits or on the ground is objectionable from the standpoint of odors produced and the danger of contamination of ground waters. Inhibition of plant growth has been observed when large amounts of whey were disposed of on land. However, when whey has been placed on the land in dilute amounts there were no adverse effects on plant growth. Sharratt et al. (1962a) studied the disposal of whey on land, and the possible use of whey as a source of plant nutrients. It was found that from the standpoint of disposal, the whey had to be spread over large areas of land to avoid harmful effects on plant growth. If more than 4 acre inches of whey was added to the soil during one season sufficient salts accumulated to temporarily inhibit plant growth (Sharratt et al. 1962b). It was also found that the whey had little effect on the pH of soils which were nearly neutral, but when added to soils with a pH of 5.0 to 5.5, whey temporarily increased the acidity to a point injurious to plant growth. The pH changes were due to the metabolic activity of microorganisms acting upon the highly fermentable whey solids. While the whey had little effect on the growth of alfalfa, it stimulated the growth of grasses during the second season of growth after application. Although the whey benefited the physical structure of the soil, it was found to add very little nitrogen, potassium and phosphorus. Midgley (1962) stated that because of its low nutrient content, whey is not a profitable proposition for farmers to use as source of plant nutrients, except for farmers near cheese factories, where some satisfactory arrangement may be worked out.

The use of stabilization ponds built specifically to handle whey wastes has been attempted, but has not been too successful. As has already been mentioned, city sewage stabilization ponds do not function properly when large quantities of whey are disposed of into the ponds. If such systems are to be free from noxious odors, they must be operated aerobically, and to do this large areas of land and great quantities of water to dilute the wastes are required.

Special sewage treatment plants to handle whey wastes have been suggested. Such plants could be designed for complete treatment of the whey or for a pre-treatment, to lower the BOD of the wastes prior to discharge into the city sewage system. If the whey wastes are to be discharged into city sewage systems, a practical method of pre-treatment of the wastes must be used (Bachmeyer, 1947). Jasewicz and Porges (1958) carried out a method of aerobic treatment of dilute whey wastes in the laboratory. They observed that with pure whey wastes a nitrogen source had to be added to get proper growth of the aerobic organisms. It was also found that when diluted whey wastes were aerated in the presence of a nitrogen rich sludge, no nitrogen supplement was needed. Further work by Porges and Jasewicz (1959) showed that with this aeration method, an average of 75% of the influent whey chemical oxygen demand (COD) was oxidized. They stated that with proper removal of sludge from the effluent a purification of 97% was possible. Ingram (1961) found that by passing whey wastes through a deep trickling filter, the BOD of these wastes could be reduced, depending on the

loading of the filter. It was found that the loadings of organic waste had to be kept below 1,380 lb/day/cu. ft. for pretreatment units and below 690 lb/day/cu. ft. for 65-70% removal of the BOD.

Returning the whey to farmers for feeding is helpful only to a limited extent. Since whey is essentially low in nutrients, large quantities must be transported to the farms in order to obtain enough nutrients for feed uses. The transportation increases the cost of a material already low in nutrient which makes whey unattractive to most farmers as a feed.

Utilization. Since the handling of whey as a waste product creates added costs to the cheese manufacturer and is also wasteful of raw materials, much work has been done to develop processes for the utilization of whey.

Webb and Whittier (1948) list a number of concentrated products obtainable from whey. These include plain condensed whey, sweetened condensed whey and dried whey. The concentrated products can be used in the manufacture of various food products such as soups, candies and baked foods. Since whey is high in lactose this sugar can be concentrated and purified, and sold commercially. Most of these products utilize only a small portion of the overall supply, and do not add materially to the amount of whey utilized annually.

A number of fermentations have been carried out using whey as a substrate. Wix and Woodbine (1958) list a number of fermented whey beverages. These products include alcoholic whey beer, malted whey

beer, whey malt beer and whey nutrient beer. Fermented whey beverages have not found widespread use outside of certain geographical areas, and contribute little to the utilization of surplus whey.

There are a number of bacterial fermentations which have been developed using whey as a substrate. According to Wix and Woodbine (1958) the most important organism used in bacterial fermentation of whey is Clostridium acetobutylicum which yields acetone and butanol as end products. It has been reported that the organism is capable of good lactose utilization, while producing appreciable levels of the solvents acetone and butanol. Whey contains 1.7 micrograms of riboflavin per milliliter, the organism involved in the acetone-butanol fermentation also produces good yields of riboflavin while producing useful yields of the solvents. The solvents can be removed by distillation and the remaining liquid dried to a riboflavin rich powder.

Lactic acid can be produced from whey using Lactobacillus bulgaricus (Wix and Woodbine, 1958). A process involving production of lactic acid by L. bulgaricus and subsequent neutralization of the acid with anhydrous ammonia has been reported by Arnott (1958). The end product is ammonium lactate. It was suggested that the product be used as a high nitrogen feed supplement for ruminants, or as a source of nitrogen for use as a fertilizer.

Wix and Woodbine (1959a,b) studied the use of molds for the production of fat from whey. It was found that in order to get good fat production a low nitrogen to carbon ratio was required. It was

also found that by adding small amounts of nitrogen, fat production was enhanced.

Yeasts have been grown in whey to produce high protein feed supplements. Graham et al. (1953a,b,c) reported results of experiments to increase the food value of whey by yeast cell production. In the initial experiment four yeast cultures were studied, Torula cremoris, Candida krusei, Torula utilis and Torula utilis var. thermophilus. Torula utilis was found to show the most promise. It was also found that nitrogen added in the form of inorganic ammonium salts or urea at levels of 2.5 to 3.0 grams per liter of whey stimulated growth. The production processes were carried out first on a laboratory scale and later on a pilot plant scale. After forty-eight hours the yeast cells were dried. Analysis of the dried powder showed that the material was 38.5% protein, 12.1% fat, 2.5% calcium and 2.2% phosphorus compared to values of 12.2% protein, 0.8% fat, 1.0% calcium and 1.6% phosphorus for dried whey powder.

Wasserman et al. (1958, 1960a,b,c, 1961a,b) investigated the possibility of using the yeast Saccharomyces fragilis to produce an animal feed supplement from whey. They found that the addition of 0.5% $(NH_4)_2SO_4$, 0.5% K_2HPO_4 and 0.1% yeast extract were necessary for good growth. They also found that maximum growth was obtained when the medium was adjusted to an initial pH of 5.0 to 5.7. When a large inocculum was used (about 2×10^9 cells/ml) the fermentation was carried out in 3 to 4 hours. Best growth was attained when oxygen was added

to the medium at the rate of about 110 ml O_2/liter of media, per minute. It was also found that S. fragilis utilized about 25% of the total whey nitrogen. They found that the usable nitrogen was available only from the non-coagulable nitrogen fraction and that the acid and heat precipitable proteins were not broken down by the actively growing yeast. In pilot plant studies yields of about 0.42 lbs. of yeast were obtained per pound of lactose, or about 0.165 lbs. of yeast per pound of whey. After growth of the yeast culture was complete, the cells were concentrated using a centrifugal separator, and the material dried. The protein content of the dried material was found to be about 50%. Eight of the ten essential amino acids were present in significant quantities as follows:

Lysine	53.2 ugm/gm dry material
Arginine	35.0
Histidine	19.2
Threonine	27.6
Valine	28.4
Methionine	7.8
Isoleucine	25.0
Phenylalanine	25.0

The dried material was also found to contain high amounts of many of the B Vitamins. Choline was especially high with 6.7 mg/gm of cells. Niacin was present at a level of 288 ugm/gm of cells.

It has been shown (Akin, 1961) that the yeast Trichosporon cutaneum when grown in a whey substrate has a lower amino acid content, but produces a higher yield of cells than S. fragilis. Akin (1961) suggested that T. cutaneum might also be a suitable organism for the production of protein using whey as a substrate.

Vitamin B_{12}. Porges (1959) suggested that whey may possibly serve as a substrate for Vitamin B_{12} production under the proper conditions. The vitamin has been observed in activated sewage sludge and in sludge containing dairy wastes (Hoover et al., 1951; Hoover et al., 1952). It was found that dried sludge, used as a commercial fertilizer, contained 3.5 to 4.0 mg of vitamin B_{12} per kg of sludge. A study of the distribution of the vitamin in activated sludge from municipal sewage treatment plants indicated that part of it was derived from the raw sewage, and part of it was synthesized by microbial activity in the aeration tanks. It was suggested that the dried sludge might serve as an animal feed supplement for vitamin B_{12}.

Davidov and Rykshina (1960) investigated the possibility of enriching the vitamin B_{12} content of skim milk and buttermilk through the action of microorganisms. They added 0.2 mg/liter of $CoCl_2$ to buttermilk, and through the action of acetic acid bacteria reported yields of 51.0 to 128.6 µgm/liter of vitamin B_{12}. They also reported that by using propionibacteria and skim milk supplemented with the precursor, 5, 6-dimethylbenzimidazole, good levels of vitamin B_{12} were attained.

Vitamin B_{12}, the anti-pernicious anemia factor, or animal protein factor was isolated and identified in 1948. Prior to 1948, liver extracts were used to treat pernicious anemia. It is now known that liver is high in vitamin B_{12}. Since its isolation, vitamin B_{12} has been found to be produced by a wide range of microorganisms (Smith 1951,

Prescott and Dunn 1959). The vitamin is presently thought to be synthesized almost exclusively by microorganisms (Smith 1960). Whenever the vitamin is found in plant or animal materials it can usually be traced back to organisms growing in water, soil or the digestive tract of animals. The importance of microbial synthesis of vitamin B_{12} was summarized by Smith (1951),

> It seems probable that the only primary source of Vitamin B_{12} in nature is the metabolic activity of microorganisms; there is no convincing evidence for its elaboration in tissues of higher plants or animals. It is synthesized by a wide range of bacteria and actinomycetes, though apparently not to any extent by yeasts or fungi.

After the identification of vitamin B_{12}, the vitamin was first obtained as a side product of antibiotic producing processes (Hester and Ward 1954, Perlman 1959). Some of the first organisms known to produce vitamin B_{12} were Flavobacterium solare, Streptomyces griseus (Mervyn and Smith 1964), Streptomyces aureofaciens and Streptomyces fradiae (Perlman 1959). Since vitamin B_{12} is essential in the diets of swine and poultry, the demand for vitamin B_{12} feed supplements grew steadily after its discovery. It then became economically feasible to produce the vitamin in processes in which it was the main product, and several commercial processes for the production of vitamin B_{12} were developed. Since it had been found that many of the Streptomyces species were producers of vitamin B_{12}, many of the early processes utilized several of these organisms (Hester and Ward 1954). Hall et al (1953) investigated vitamin B_{12} production by Streptomyces olivaceus.

The studies were conducted using shake flasks and deep tank fermentors with aeration. They found that in media containing proteinaceous material, such as distillers solubles, glucose, $CaCO_3$ and cobaltous ion, about 1.5 µgm of vitamin B_{12} per ml were formed in deep tank fermentors. Appreciable amounts of other B vitamins were also obtained. In 1954 Hester and Ward reported a commercial process for manufacture of vitamin B_{12} using Streptomyces olivaceus.

In 1952 Leviton and Hargrove reported vitamin B_{12} activity in cultures of propionic acid bacteria. They state that all species of the genus Propionibacterium, when grown under the proper conditions, will produce vitamin B_{12} activity. In this study the organism, Propionibacterium freudenreichii, gave yields as high as 3.0 mg/liter. It was also found that almost all of the vitamin B_{12} activity was retained in the bacterial cell.

Since the work of Leviton and Hargrove many additional publications have appeared in the literature concerning production of vitamin B_{12} by propionibacteria (Grant, 1960; Goncharova et al., 1958; Perlman and Barrett, 1958; Perlman et al., 1961; Speedie and Hull, 1960; Sudarsky and Fischer, 1957). At the present time the organisms Propionibacterium freudenreichii and Propionibacterium shermanii are in general use in the United States for the commercial production of vitamin B_{12} using substrates other than whey. These organisms are being used because of their ability to produce substantially higher yields than most of the Streptomyces species (Hall 1964).

Production Media. Most of the media for production of vitamin B_{12} using propionibacteria contain yeast products to supply various growth factors (Perlman et al., 1961). The main carbon sources have been beet molasses, invert sugar, glucose and maltose. Leviton and Hargrove (1952) and Hargrove and Leviton (1955) used lactic acid as a carbon source for P. freudenreichii and P. shermanii. They introduced whey powder containing 80% lactose and fermented it to lactic acid using Lactobacillus casei. The lactic acid was then used as a carbon source for the propionibacteria. Sometimes the yeast products supply significant quantities of nitrogen, but additional nitrogen is usually added as ammonium hydroxide or soybean oil meal. Corn steep liquor, sewage and penicillin mycelia have also been used to supply nitrogen and various growth factors (Mervyn and Smith, 1964).

Besides a carbon source, nitrogen source, and a source of growth factors, most media for the production of vitamin B_{12} have contained cobalt, since cobalt is an essential part of the vitamin B_{12} molecule. Salts such as cobalt nitrate and cobalt chloride are suitable sources of cobalt. Speedie and Hull (1960), using various species of Propionibacterium, supplied cobalt in the range of 2.5 to 5 ppm in the form of inorganic salts of cobalt. Hargrove and Leviton (1955) used 1.1 mg/liter (1.1 ppm) of cobalt with the organisms P. freudenreichii and P. shermanii.

Vitamin B_{12} Analogues. It is known that in addition to the so-called true vitamin B_{12} two analogues of the vitamin exist,

pseudovitamin B_{12} and Factor A. It has been shown that pseudovitamin B_{12} contains adenine and Factor A contains 2-methyladenine attached to the B_{12} moiety instead of 5, 6-dimethylbenzimidazole as found in the so called true vitamin B_{12} (Smith, 1960). The chemical structure of vitamin B_{12} is shown in Figure I. The purine containing analogues have full B_{12}-like activity in certain microorganisms that require vitamin B_{12}. The analogues show less activity toward certain other microorganisms that are more specific in their vitamin B_{12} requirement and have little or no activity toward higher animals. The benzimidazole analogues show activity in all organisms tested including higher animals and humans suffering from pernicious anemia. An additional substance designated as Factor B has also been observed in fermentation liquors and feces. This factor has been identified as vitamin B_{12} without the nucleotide portion (Smith, 1960), and is probably an intermediate in the formation or degradation of the vitamin.

Synthesis of Vitamin B_{12}. In recent studies by Speedie and Hull (1960) it was shown that there are two stages in the synthesis of vitamin B_{12} by propionibacteria. The first is an anaerobic stage in which the organisms evolve CO_2 and produce mainly Factor B. The second stage is a microaerophilic to aerobic stage in which Factor B is converted to vitamin B_{12}. The aeration may be dispensed with, but under anaerobic conditions the precursor 5,6-dimethylbenzimidazole must be added to insure the formation of true vitamin B_{12}.

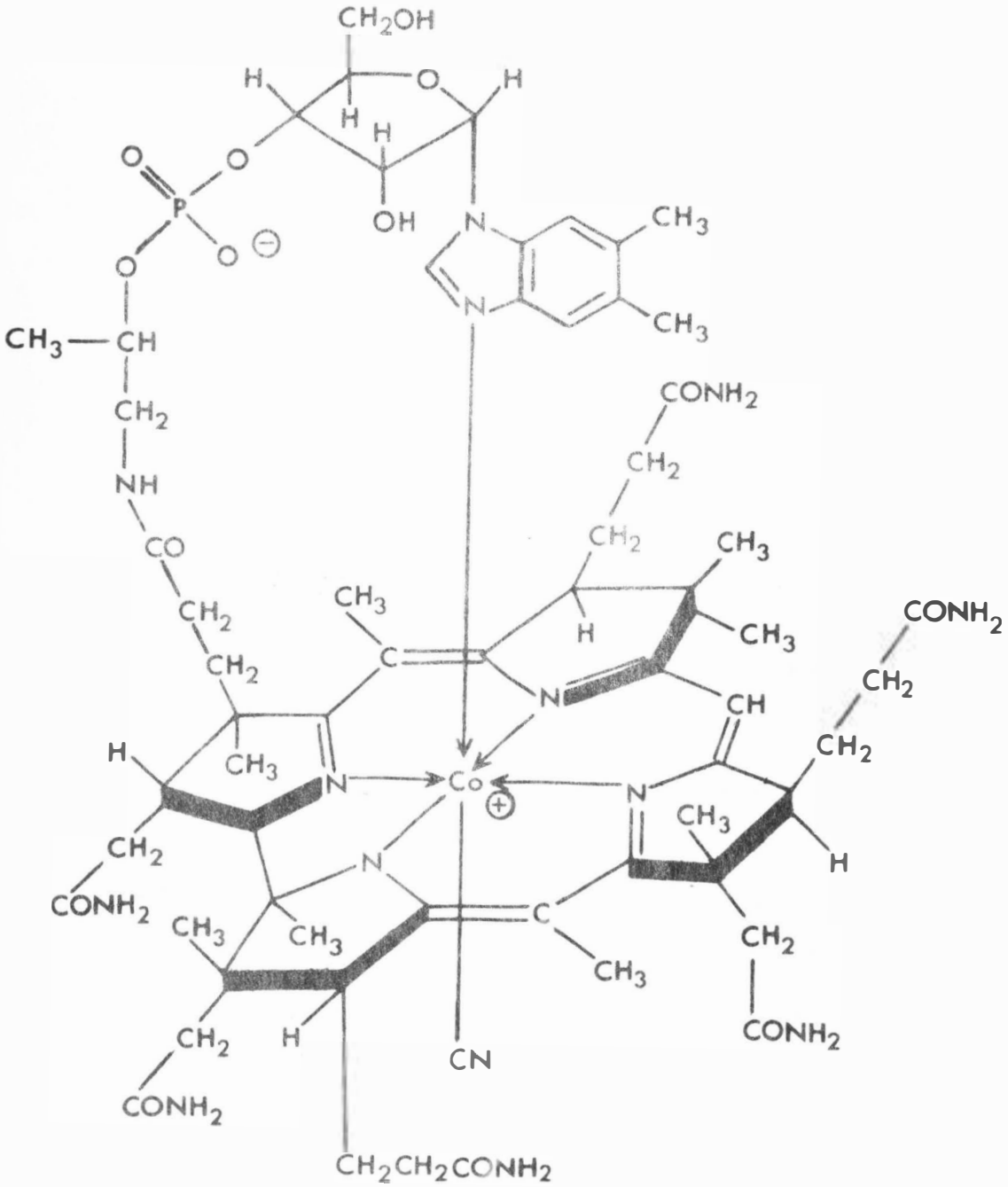

Figure 1. The 5,6 - dimethylbenzimidazole form of Vitamin B_{12}. (Conn, E. E. and P. K. Stumpf. Outlines of Biochemistry. John Wiley and Sons, Inc. 1963. page 151.)

Speedie and Hull (1960) have also shown that 80-100% of the total cell growth takes place during the first or anaerobic stage of the process.

Assay of Vitamin B_{12}. Levels of vitamin B_{12} are usually determined using microbiological assay techniques. While these techniques are widely used, they are subject to certain disadvantages. The main disadvantage of the microbiological assays is that the test organisms will respond to substances other than vitamin B_{12}.

A number of organisms have been used to assay for vitamin B_{12}. These include Lactobacillus leichmannii, Escherichia coli mutants, Euglena gracilis and Ochromonas malhamensis. Lactobacillus leichmannii ATCC 7830 is currently the organism used in approved methods of the United States Pharmacopea and Association of Official Agricultural Chemists.

Lactobacilli show a growth response to analogues containing purines in the nucleotides but do not respond to Factor B. Since Lactobacillus leichmannii shows a high sensitivity to vitamin B_{12} many of the interfering substances can be diluted out (Mervyn and Smith, 1964). The E. coli mutant will respond to Factor B and analogues containing purine and benzimidazole in the nucleotide. Euglena gracilis is very sensitive to vitamin B_{12}, but has the same drawbacks as E. coli in responding to B_{12} analogues, with the added disadvantages of requiring a long incubation period and illumination. The organism

Ochromonas malhamensis is the most specific and highly sensitive in its growth response, and responds in much the same manner as chicks and animals. In recent years isotope dilution assays for vitamin B_{12} have been developed. These methods involve the use of cyanacobalamin labeled with cobalt 60 (Mervyn and Smith, 1964).

MATERIALS AND METHODS

MATERIALS

Organisms. The organism used for the production of vitamin B_{12} in this study was Propionibacterium shermanii, American Type Culture Collection (ATCC) strain number 13673. The organism was obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

The organism was chosen for this study for two main reasons, (1) it is a known producer of vitamin B_{12} which is used commercially to obtain the vitamin, and (2) its ability to use lactose as a carbon source. According to Bergeys Manual of Determinative Bacteriology, seventh edition, P. shermanii resembles P. freudenreichii in every respect except the ability to ferment lactose. The organism produces carbon dioxide and propionic acid during its growth, grows anaerobically but is aerotolerant. It was isolated from dairy products which are its natural habitat.

The organism was carried in stock culture in deep agar tube stabs. The medium used to carry the organism had the following composition:

Glucose	3.0 gm
Yeast Extract	2.0 gm
Cysteine HCl	0.05 gm
$CaCO_3$	0.50 gm
Agar	1.7 gm
H_2O	100 ml

The test organism used in the vitamin B_{12} assay was Lactobacillus leichmannii (ATCC 7830). The organism was carried in stab cultures using Difco Bacto B_{12} Culture Agar U.S.P. which had the following composition:

Tomato Juice	10.00 ml
Proteose Peptone No. 3, Difco	0.75 gm
Bacto-Yeast Extract	0.75 gm
Bacto-Dextrose	1.00 gm
Monopotassium Phosphate	0.20 gm
Sorbitan Monooleate Complex	0.01 gm
Bacto-Agar	1.50 gm
H_2O	100 ml

Substrate. The substrate used in this study was dried whey reconstituted with deionized water. Dried whey was used in this study rather than fresh liquid whey because of the ease of handling and storing and because a constant supply of liquid whey was not available.

Initially, it was decided to begin the study using three sources of dried whey to determine if variations between sources of whey would have any effect on vitamin production. The dried whey was obtained from Foremost Dairies, Appleton, Wisconsin (Source A); Bongards Creameries, Bongards, Minnesota (Source B); and Valley Queen Dairy, Milbank, South Dakota (Source C).

Since it was known from the literature (Mervyn and Smith, 1964) that yeast products stimulate the growth of the propionibacteria it was decided to supplement the whey with yeast extract. The yeast extract used was Bacto-Yeast Extract obtained from Difco Laboratories,

Detroit, Michigan. Bacto-yeast extract is the water soluble portion of autolyzed fresh yeast. It is a source of B vitamins and other growth promoting substances.

Cobalt was added to the medium as an aqueous solution of cobalt chloride. The cobalt stock solution was made by dissolving sufficient $CoCl_2 \cdot 6H_2O$ in deionized water to give a solution containing 1 gram of cobalt per 100 ml of stock solution. The stock solution was sterilized by autoclaving at 121°C. for 15 minutes. The desired levels of cobalt in the culture flasks were obtained by aseptically adding varying amounts of the stock solution to the sterile media.

The 5,6-dimethylbenzimidazole precursor was obtained from Dr. H. C. Friedmann of the Department of Biochemistry, the University of Chicago. The material when received was impure, and was purified by recrystallization from boiling water. A stock solution of the precursor was prepared by dissolving 0.5 grams of the material in 1000 ml of deionized water. The stock solution was sterilized by autoclaving, and added aseptically to the appropriate flasks. The levels of cobalt and 5,6-dimethylbenzimidazole that were used are given in the Experimental Design section.

Culture Vessels and Preparation of Substrate. The fermentations were carried out in 2 liter flasks containing 1.5 liters of medium. The flasks were closed with rubber stoppers that had been fitted with two pieces of glass tubing. One piece of glass tubing was drawn to a capillary point which extended into the medium to within 1 to 2 cm of

the bottom of the flask. This tubing was used for addition of gases to the flasks. The capillary point was used to produce small bubbles of the gas in the medium. The other piece of glass tubing, which was used to allow escape of gases from the flasks, extended just through the rubber stopper and was plugged with cotton. The cotton was used to prevent cross contamination from one flask to another.

The whey solutions containing the proper amount of dried whey to give the desired solids levels for 1.5 liters were made up to 1 liter in the culture flasks. The levels of whey used are given in Experimental Design section. The yeast extract was made up separately because of solubility reasons, with the desired amount of yeast extract for 1.5 liters contained in 350 ml of deionized water. The amounts of yeast extract used are given in the Experimental Design section. This amount (350 ml) of yeast extract solution was then added to each one liter of whey solution and the pH adjusted to the range 7.0 to 7.5. This gave a volume of 1350 ml of whey and yeast solution in each flask. The remaining part of the volume (150 ml) was made up from the inocculum at the time of inocculation for a final volume of 1500 ml. The flasks containing whey and yeast extract were covered with aluminum foil and autoclaved at 121°C for 20 minutes. Cobalt and 5,6-dimethylbenzimidazole were added aseptically after sterilization. The stopper assemblies were sterilized separate from the flasks and the apparatus assembled aseptically after inocculation. The flasks were joined together in series with rubber tubing that had

been attached to the stopper assemblies prior to autoclaving. From four to six flasks were joined in one series (Figure II). The series of flasks was then connected to the carbon dioxide source and carbon dioxide added to the flasks to insure anaerobiosis. The CO_2 was added to all flasks during the first one-half of the process. Under the system used, a sterile cotton filter was inserted between the CO_2 source and the first flask. The carbon dioxide was bubbled into the first flask, and the atmosphere of the first flask moved to the second flask and so on down the line. In this way maximum use of the CO_2 was attained.

PROPAGATION AND ANALYTICAL METHODS

Preparation of Inocculum. To prepare the inocculum, a loop full of cells was taken from the stab culture and placed in 10 ml. of a broth solution consisting of 0.5% lactose, 0.5% yeast extract and 0.5% peptone. This was incubated for 48 hours at 29°C. The contents of the broth culture were transferred to 140 ml of sterile 6% whey and 0.5% yeast extract and incubated an additional 48 hours at 29°C. The complete whey culture (150 ml) was used as inocculum for one culture flask. When added to the flask, the inocculum brought the final volume in the flask to 1500 ml, and constituted a 10% inocculum.

Culture Conditions. After inocculation, the flasks were weighed and the weight recorded. The flasks were then connected to the carbon dioxide source and the contents incubated at 29°C. Daily pH

Figure II. Flasks, Containing Culture Material, Connected in Series as Used in the Study

measurements were made using a Beckman "Zeromatic" pH meter. A 10 ml sample was taken from each culture each day for the purpose of pH measurements. Each day the pH was found to have dropped to an acid pH, usually 4.9 to 5.9, because of the production of propionic acid by the organism. The culture was kept in the logarithmic phase of growth as long as possible by daily pH adjustment to the pH range of 6.5 to 7.5 using 5N NH_4OH. Besides controlling pH, the nitrogen of the NH_4OH was available to the organism for growth.

At the end of the incubation period the flasks were again weighed, and a weight difference determined. This difference was used to correct the vitamin B_{12} assays to a constant weight basis for all cultures, since some cultures gained weight and some lost weight depending upon their position in the series. The differences were due to losses from evaporation and sampling and the gain from the addition of materials. The corrections for weight changes in the cultures were made as follows:

$$\frac{1}{\text{Initial net weight}} \times \text{initial weight} \pm \text{weight change} \times \text{assay readings} = \text{Corrected assay}$$

Two 30 ml subsamples were taken from each culture. Each sample was taken in a representative manner in three 10 ml portions using a sterile 10 ml pipette. The samples were immediately frozen and stored at -70°C until vitamin assays could be performed on the material. The same samples were also used for lactose determinations.

It was found when the cultures were 168 hours old that the lactose level was usually less than 2.0%. At this point the incubation period was terminated and samples taken. This is in accord with recent studies of Speedie and Hull (1960). The majority of the present studies were carried out using a culture time of 168 hours, however, additional work was done using longer culture times, to determine if additional vitamin B_{12} would be produced. These times are given under the Experimental Design section.

Vitamin B_{12} Bioassay. The vitamin B_{12} content of the fermentation samples was determined using a modified U.S.P. microbiological assay technique (Difco Laboratories; Pharmacopea of the United States, 1960).

The vitamin B_{12} reference standard used in the assay was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, as a sterile, water solution of vitamin B_{12} in sealed glass vials. Each vial contained 1 ml of standard solution which had a vitamin B_{12} activity of 15 µgm/ml. A standard stock solution of vitamin B_{12} containing 1 µgm/ml was made by transferring the contents of one vial (15 µgm) to a large screw cap test tube and adding 14 ml of 25% ethanol. The stock solution was made fresh every two weeks and stored at 3 to 5°C.

A standard curve was prepared each time that a vitamin assay was made. To prepare the standard curve 1 ml of standard stock solution was diluted to 100 ml with double distilled water. This solution

contained vitamin B_{12} activity of 10 mμgm/ml. One milliliter of the diluted solution was further diluted to 200 ml with double distilled water, for a final vitamin B_{12} activity of 0.05 mμgm/ml. The standard curve was constructed by using varying amounts of the final solution. To construct the standard curve the following levels of vitamin B_{12} were used; 0.0, 0.025, 0.05, 0.075, 0.100, 0.125, 0.150, 0.200, and 0.250 mμgm respectively per assay tube. Sufficient double distilled water was added to the vitamin solution in each tube to make 5 ml. The assay broth was made double strength and 5 ml added to each tube for a final volume of 10 ml of single strength broth containing the vitamin. The assay broth used was Difco Bacto-B_{12} Assay Broth U.S.P. and had the following composition per liter:

Bacto-Vitamin Free Casamino Acids	15 gm	P-Aminobenzoic Acid	2 mg
Bacto-Dextrose	40 gm	Calcium Pantothenate	1 mg
Bacto-Asparagine	0.2 gm	Pyridoxine Hydrochloride	4 mg
Sodium Acetate	20 gm	Pyridoxal Hydrochloride	4 mg
Ascorbic Acid	4 gm	Pyridoxamine Hydrochloride	800 μgm
L-Cystine	0.4 gm	Folic Acid	200 μgm
DL-Tryptophane	0.4 gm	Monopotassium Phosphate	1 gm
Adenine Sulfate	20 mg	Dipotassium Phosphate	1 gm
Guanine Hydrochloride	20 mg	Magnesium Sulfate	0.4 gm
Uracil	20 mg	Sodium Chloride	20 mg
Xanthine	20 mg	Ferrous Sulfate	20 mg
Riboflavin	1 mg	Manganese Sulfate	20 mg
Thiamine Hydrochloride	1 mg	Sorbitan Monooleate Complex	2 gm
Biotin	10 μgm		
Niacin	2 mg		

The tubes were sterilized by autoclaving at 121°C for 5 minutes. The test tubes had been previously sterilized by autoclaving at 121°C for 20 minutes before the addition of vitamin and medium.

To prepare inocculum for the assay, 10 ml of Difco-Bacto B_{12} Inocculum Broth U.S.P. was inocculated with a 16 to 24 hour stab culture of Lactobacillus leichmannii (ATCC 7830). The inocculum broth had the following composition:

Tomato Juice	10.00 ml
Proteose Peptone No 3, Difco	0.75 gm
Bacto-Yeast Extract	0.75 gm
Bacto-Dextrose	1.00 gm
Monopotassium Phosphate	0.20 gm
Sorbitan Monooleate Complex	0.01 gm
H_2O	100 ml

The broth culture was incubated 24 hours at 36°C ± 0.5°C and the cells so obtained were washed four times with sterile assay broth. After the last washing the cells were resuspended in 10 ml of sterile assay broth. A 0.1 ml portion of the suspension was then transferred to an additional 10 ml of sterile assay broth for a 1 to 100 dilution, and thoroughly mixed to obtain a homogeneous suspension. This constituted the inocculum and was used at the rate of one drop per assay tube. After inocculation the tubes were incubated for 18 hours at 36°C ± 0.5°C. The growth of the test organism was measured turbidimetrically with a Bausch and Lomb "Spectronic 20" colorimeter at a wave length of 540 mμ. The optical density (OD) of the samples was determined and plotted against vitamin B_{12} concentration to obtain the standard curve. A typical standard curve is shown in Figure III.

To prepare the unknown samples for assay, the samples were first extracted using the U.S.P. extracting solution (Pharmacopea of the United States, 1960). The samples were extracted by placing 1 ml of

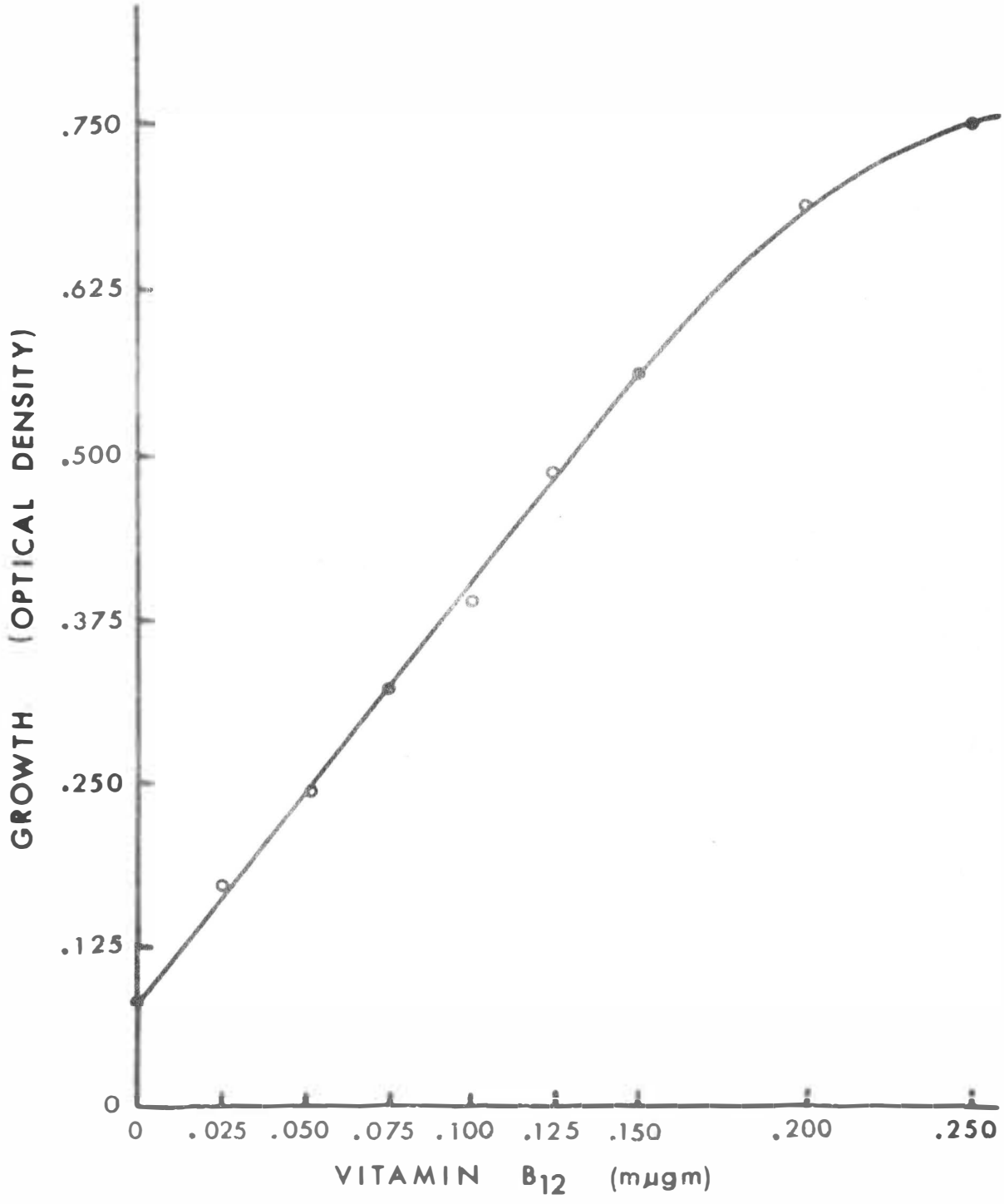

Figure III. Typical standard curve for Vitamin B_{12} assay using Lactobacillus leichmannii ATCC 7830.

unknown sample in a small flask, adding 24 ml of extracting solution and autoclaving at 121°C for 10 minutes. The extracting solution was prepared with 100 ml of double distilled water and contained the following constituents:

Disodium phosphate	1.29 gm
Anhydrous Citric Acid	1.10 gm
Sodium Metabisulfite	1.00 gm

The pH of the extracting solution prepared in the above manner was 4.2 to 4.5.

Following extraction, the samples were sufficiently diluted with distilled water to be readable in the most linear part of the standard curve. One milliliter of the diluted sample was placed in an assay tube and 4 ml of double distilled water added to bring the volume to 5 ml. Five milliliters of double strength assay broth was added to each tube to obtain a total volume of 10 ml. The tubes were sterilized by autoclaving at 121°C for 5 minutes. The samples were inocculated, incubated and the growth determined in the same manner as was described for the standard curve. The samples were done in duplicate and the readings were averaged. The vitamin B_{12} content of the samples was then determined from the standard curve and the dilution factor.

Lactose Determination. The levels of lactose in the substrate were determined at various times after inocculation using an anthrone method suitable for use with milk (Loewus, 1952; Morris, 1948). The anthrone reagent was prepared by dissolving 0.2 grams of anthrone in

100 ml of 95% H_2SO_4. A 2 ml portion of the substrate was treated with 5 ml of 5% Trichloroacetic acid to precipitate the proteins. This mixture was then diluted to 100 ml with distilled water and clarified by filtration using Whatman number 5 filter paper. A 2 ml portion of the clear filtrate was again diluted to 100 ml for a final dilution of 1:2500. Three milliliters of the final dilution were placed in a colorimeter tube with 6 ml of anthrone reagent. At the same time a 100 µgm lactose standard and a distilled water blank were also included. The color was developed by placing the tubes in a boiling water bath for 3 minutes. After cooling, the optical density of samples was determined using a Bausch and Lomb "Spectronic 20" colorimeter at a wave length of 620 mµ. The color density follows Beer's Law, and the lactose levels were determined from the proportional relationships stated in Beer's Law.

EXPERIMENTAL DESIGN

Determination of Whey Levels and Optimum Culture Times. An initial study was conducted, to determine (1) if there would be substantial differences between whey sources (2) the optimum time of fermentation and (3) the range of solids levels to be studied.

Whey from each of the sources was reconstituted to give solids levels of 6%, 12% and 18%. Yeast extract was used at a level of 0.5%, cobalt at a level of 20 ppm and the precursor, 5,6-dimethylbenzimidazole, was used at a level of 15 ppm which was added in three equal

portions. The first addition of precursor was made 12 hours after inocculation, the second addition 48 hours later and the third addition 48 hours after the second addition.

Carbon dioxide was added to the cultures during the first 84 hours of growth. The medium was then aerated for an additional 84 hours at a rate of 75 to 100 ml of air per liter per minute as measured by water displacement.

Foaming was a persistent problem and was controlled by the aseptic addition of six drops of a 1:3 aqueous dilution of Dow Corning Antifoam "C," a food grade silicone defoamer, to each flask.

Samples for vitamin assay and lactose determination were taken at 168 hours, 190 hours, 245 hours and 266 hours. The vitamin B_{12} and lactose levels were determined in the manners previously described.

Determination of Optimum Levels of Whey and Yeast Extract. From a study of the data obtained in the initial study and the work of Speedie and Hull (1960) it was decided to use somewhat lower levels of whey solids. The levels of whey chosen were 6%, 8% and 10%. Since the optimum level of yeast extract in relation to whey solids was not known, it was also decided to study three levels of yeast extract. The yeast extract is used mainly to supply certain vitamins and growth factors which are normally needed in small amounts, therefore the levels of yeast extract used were 0.5%, 1.0% and 1.5%.

The experiment was set up as a completely randomized block design of a factorial type (3x3x2) with three levels of whey solids,

three levels of yeast extract and two replications. An analysis of variance was performed on the data so obtained. The design of the experiment is shown in Table 1.

Table 1. Combinations of Levels of Whey and Yeast Extract Studied at 20 ppm of Cobalt, 15 ppm Precursor and 200 ml of air/liter/min.

Percent Yeast Extract	Percent Whey Solids		
0.5	6	8	10
1.0	6	8	10
1.5	6	8	10

In this experiment whey solids and yeast extract at three levels were studied; precursor, cobalt and aeration which were to be studied later were held constant.

Since cobalt is necessary for the formation of vitamin B_{12}, it was decided to fix the level of cobalt sufficiently high to insure an adequate amount. The level of cobalt was fixed at 20 ppm for this experiment, since it was not thought that this amount would be inhibitory to the growth of the organsim.

To insure an adequate amount of precursor, it was decided to fix the level of 5,6-dimethylbenzimidazole at 15 ppm (Perlman, 1965) added in three equal portions. Aeration was supplied during the last

one-half of the experiment at the rate of 200 ml of air per liter per minute. Foaming was controlled in the manner previously described.

From the results of the initial studies, the fermentation time was set at 168 hours. Carbon dioxide was supplied during the first 84 hours and air the last 84 hours.

Inocculation, pH adjustment and sampling were done in the manner described above.

Determination of Optimum Levels of Cobalt, Precursor and Aeration. The experiment was set up as a completely randomized block design of a factorial type (3x3x3x2) with three variables, three levels of each variable and two replications. The levels of cobalt were 5 ppm, 15 ppm and 25 ppm. The levels of 5,6-dimethylbenzimidazole precursor were zero, 10 ppm and 15 ppm. Aeration was studied mainly to observe the effect of aeration over no aeration. The levels of aeration were zero, 215 and 1000 ml of air/liter/minute measured by water displacement. On the basis of previous studies, the whey level was fixed at 10% and the yeast extract level at 1.5%.

Carbon dioxide was added to all flasks during the first 84 hours of culture time, with aeration beginning in the appropriate cultures at 84 hours after inocculation. Carbon dioxide was added for the complete incubation period (168 hours) to the cultures of the zero air treatment. Foaming was controlled in the manner previously described. The statistical design of this study is shown in Table 2.

Table 2. Combinations of Levels of Cobalt, Precursor and Aeration Studied at 10% Whey and 1.5% Yeast Extract.

No Aeration		Low Aeration*		High Aeration**	
Precursor (ppm)	Cobalt (ppm)	Precursor (ppm)	Cobalt (ppm)	Precursor (ppm)	Cobalt (ppm)
0	5	0	5	0	5
10	5	10	5	10	5
15	5	15	5	15	5
0	15	0	15	0	15
10	15	10	15	10	15
15	15	15	15	15	15
0	25	0	25	0	25
10	25	10	25	10	25
15	25	15	25	15	25

*215 ml air/liter/minute
**1000 ml air/liter/minute

Again the fermentation was conducted for 168 hours in the same manner as was done previously. Two 30 ml subsamples were taken from each culture, frozen and stored at -70°C as previously described.

Growth Response of the Organism to Optimum Conditions. Growth studies were conducted to show the rate of growth, rate of vitamin formation, rate of lactose utilization and pH changes in the whey. It was desired to know if these changes were comparable to that which had been reported in the literature (Speedie and Hull, 1960; Mervyn and Smith, 1964).

The growth rate was calculated by determining the number of viable organisms per milliliter at increasing time intervals after inoculation. Samples for growth studies were taken at the following times 0, 15, 48, 60, 72, 96, 108, and 120 hours. The bacterial numbers

in the samples were determined by plate counts using a pour plate technique. The medium used in plate counts had the following composition:

Glucose	3.00 gm
Yeast Extract	2.00 gm
Cysteine HCl	0.05 gm
$CaCO_3$	0.50 gm
Agar	1.70 gm
H_2O	100 ml

Dilutions of the samples were made using phosphate buffered dilution blanks. The dilution blanks were made by adding 1.25 ml of a KH_2PO_4 stock solution to 998.75 ml of deionized water. To make the stock solution 34 grams of KH_2PO_4 was dissolved in 500 ml of deionized water, the pH was adjusted to 7.2 with 10N NaOH and the volume made up to one liter with deionized water (Frazier and Foster, 1959). The plates were incubated for 5 days at 29°C under a carbon dioxide atmosphere, and the colonies counted.

The rate of vitamin B_{12} formation was determined by assaying, in the manner previously described, samples taken at the following times; 0, 6, 18, 30, 42, 63, 76, 98, 114, 136, 168, 192, and 216 hours after inocculation.

Lactose levels of the samples were determined using the method given on page 32. The samples for lactose analysis were taken at the following times; 0, 6, 18, 30, 42, 63, 76, 90, 114, 136, 168, 192, and 216 hours after inocculation.

The pH changes were determined at the following times during the course of the study; 0, 24, 48, 72, 99, 120, 144, 168, 192, and 216 hours.

Vitamin B_{12} and Feed Analysis of Dried Fermentation Solids. It was decided that it would be desirable to know the vitamin B_{12} activity of the fermentation material on a dry weight basis. To obtain dried material for analysis a portion of the fermentation mixture was concentrated using a Servall model SS-34 automatic centrifuge with continuous flow attachments at 18,000 rpm. The concentrated material was then dried at 98°C for one and a half hours. After grinding with a mortar and pestle, one gram of the dried material was extracted and assayed in the manner previously described. The vitamin B_{12} level was converted to a moisture free basis using the information obtained from the feed analysis. The vitamin B_{12} activity of dried unfermented whey was determined in a similar manner. The dried fermentation material was also subjected to a feed analysis. The percent moisture, ether extract, crude fiber, crude protein, ash and nitrogen free extract were determined.

RESULTS AND DISCUSSION

Determination of Levels of Whey and Optimum Culture Times. The initial studies showed no substantial differences between sources of whey. While source A exhibited a slight advantage at the 6% level, the differences were not considered great enough to warrant a study based on source differences. Table 3 gives the yields of vitamin B_{12} in micrograms per milliliter at the different solids levels for all three sources. The samples were taken at 168 hours after inocculation.

Table 3. Yields of Vitamin B_{12} for Various Whey Sources and Solids Levels.

Whey Source*	Solids Level	Vitamin B_{12} µgm/ml at 168 hrs.
A	6%	8.25
B	6%	7.50
C	6%	6.62
A	12%	6.25
B	12%	6.87
C	12%	6.56
A	18%	5.37
B	18%	5.52
C	18%	5.50

*Whey sources: Source A, Foremost Dairies; Source B, Bongards Creameries; Source C, Valley Queen Dairy.

The greatest differences between sources of whey were observed at the 6% solids level where a difference of 1.63 µgm/ml was observed between source A and source C. Since the differences were not great,

it was believed that the time available for the remaining work could be better used studying variables other than source of whey solids. It was decided to use source A in all future work.

No great differences were observed between sources of whey as to percent lactose remaining at 168 hours. Greater amounts of lactose were present in the high levels of whey solids at 168 hours after inocculation than in the low levels of whey. The levels of lactose remaining are given in Table 4.

Table 4. Percent Lactose Remaining at 168 Hours After Inocculation.

Whey Source	Solids Level	Percent Lactose Remaining at 168 Hrs.
A	18%	7.55
B	18%	7.80
C	18%	6.65
A	12%	4.60
B	12%	4.60
C	12%	3.85
A	6%	1.35
B	6%	.85
C	6%	.72

A study of the time of incubation showed that vitamin B_{12} levels did not increase appreciably beyond 168 hours of culture time. The 6% solids level was not studied beyond 190 hours since the lactose level was very low and no further changes were expected beyond this point. The 12% and 18% solids levels were not sampled at 190

hours but were sampled at 245 hours and 266 hours. These data are summarized in Table 5.

Table 5. Vitamin B_{12} Levels at Increasing Culture Times.

Whey Source	Solids Level	Vitamin B_{12} µgm/ml 168 Hrs.	190 Hrs.	245 Hrs.	266 Hrs.
A	6%	8.25	6.62	--	--
B	6%	7.50	6.71	--	--
C	6%	7.72	7.25	--	--
A	12%	6.25	--	5.87	6.93
B	12%	6.87	--	6.62	5.69
C	12%	6.56	--	6.18	6.12
A	18%	5.37	--	5.12	4.82
B	18%	5.52	--	5.50	5.37
C	18%	5.50	--	5.12	6.56

The data presented in Table 5 show no apparent practical advantage to a culture time longer than 168 hours. This is especially true at the 6% solids level, where the data in Table 4 show that the level of lactose has been depleted to a point where very little additional growth or activity would be expected.

The higher solids levels, while still containing appreciable levels of lactose, did not produce any substantial increase in vitamin B_{12} levels beyond 168 hours. A possible explanation of this is the fact that only 0.5% yeast extract was used at all solids levels. Since yeast products stimulate the growth of the organism, it is quite possible that certain growth factors found in yeast extract became

limiting, and as a result curtailed growth. However as Speedie and Hull, (1960) point out, oxygen may be added to the medium when 80% to 100% of the cell growth has occurred, this usually takes place during the first 80 hours of incubation. So even with adequate amounts of yeast extract it may be expected that most of the growth would still take place during the first 80 hours of incubation, and that there would be no advantage to a culture time beyond 168 hours.

Since the organism produces propionic acid during its growth, pH changes are an indication of the activity of the organism. Toward the end of the fermentation pH changes were markedly reduced, which indicated that the "fermentation" was completed.

From a study of the results of the initial work it was decided to investigate a lower range of whey solids levels, since it was believed that more efficient utilization of the substrate would occur at lower solids levels. It was decided to study 6%, 8% and 10% whey solids in the remaining work. This is consistent with the results of Speedie and Hull (1960) who recommend glucose or lactose levels of from 8% to 12%. A whey solids level of 10% contains approximately 8% lactose.

Determination of Optimum Levels of Whey and Yeast Extract. The levels of whey used in this study were 6%, 8% and 10% and the levels of yeast extract were 0.5%, 1.0% and 1.5%. With a low level of whey solids (6%) it was observed that a low level of yeast extract (0.5%) was adequate. The higher levels of yeast extract (1.0% and 1.5%) did not give higher yields of vitamin when used with 6% whey solids.

However, with higher levels of whey (8% and 10%) the higher levels of yeast extract (1.0% and 1.5%) were superior to the lowest level (0.5%) of yeast extract. With 8% whey solids the 1.5% level of yeast extract exhibited a slight advantage over the 1.0% level, but the differences were not great. These data are given in Table 7 and illustrated graphically in Figure IV. It is believed that either 1.0% or 1.5% of yeast extract would be adequate with 8% whey solids, but to insure an adequate supply of yeast products 1.5% could be used. With 10% whey solids, the results indicated that only 1.5% yeast extract was sufficient to produce higher yields of vitamin B_{12}. Both 0.5% and 1.0% levels of yeast extract gave lower yields of vitamin when used with 10% whey than did 1.5% yeast extract. The results of this experiment are tabulated in Table 7 and shown graphically in Figures IV and V.

An analysis of variance performed on the data demonstrated that the only significant effect was observed among the levels of yeast extract (0.5%, 1.0%, 1.5%). None of the other variables had significant effects, including percent whey and replications. There was no significant interaction between levels of whey and levels of yeast extract, nor was there significant interaction between levels of yeast extract and replications, nor between the combination percent yeast extract, percent whey and replications. The error term or variation between subsamples was extremely small. However, the interaction between percent whey and replications was significant with no apparent

Figure IV. Effect of yeast extract on Vitamin B_{12} production at three levels of whey solids.

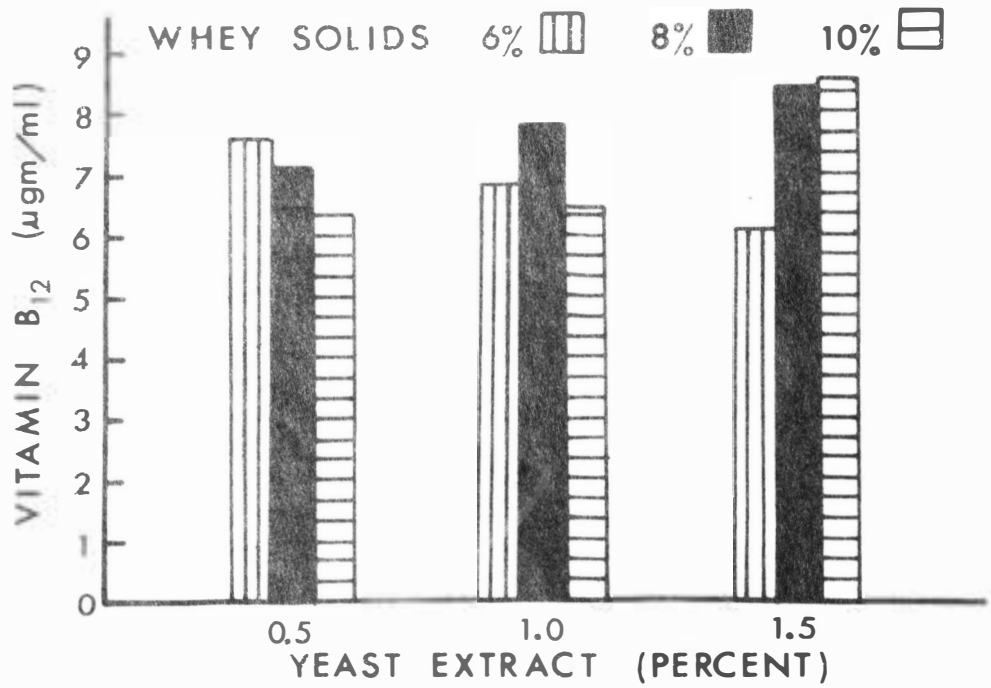

Figure V. Effect of whey solids on Vitamin B_{12} production at three levels of yeast extract.

biochemical explanation, even though the individual effects of whey and replications or the combination of whey, yeast extract, and replicates were all not significant. The analysis of variance is given in Table 6.

In the analysis of variance, (Steel and Torrie, 1960) the observed value (Fo) is compared with the tabular value (Ft). For example, in line 1, Table 6, the effect of yeast extract on vitamin B_{12} yield with 2 and 20 degrees of freedom was tested to decide whether to accept the hypothesis that there is no difference in the vitamin B_{12} concentration of the culture when it is grown in the presence of 0.5%, 1.0% and 1.5% yeast extract. The Ft value for 2 and 20 degrees of freedom is 3.49 for 5% probability. Since the Fo value is 4.60 and exceeds the expected or tabular value, 3.49, it may be concluded that the experiment provides evidence for real differences among the means for vitamin B_{12} yield when 0.5%, 1.0% and 1.5% yeast extract yields are compared. These differences are therefore said to be statistically "significant." Other experimental variables such as replications and whey solids did not have Fo values which exceed the Ft values and therefore are said to be "non-significant."

Lactose utilization by the organism under the various treatments was determined. With 6% and 8% whey almost all of the lactose was used by the organism. With these two levels of whey solids, less than 1% of the lactose remained at 168 hours after inocculation. A greater amount of lactose remained when a level of 10% whey solids was

Table 6. Analysis of Variance of Vitamin B_{12} Production by *Propionibacterium Shermanii* for Three Levels of Whey Solids and Three Levels of Yeast Extract[a] at 20 ppm Cobalt, 15 ppm 5,6-Dimethylbenzimidazole and 200 ml of Air/Liter/Minute.

Line No.	Effect	Degrees Freedom	Sum of Squares	Mean Squares	Error Testing Line	F_o[b]	F_t[b]	Significance
1	A (Yeast Extract)	2	3.1720	1.5860	9	4.60	3.49	Significant
2	B (Replications)	1	0.1134	0.1134	8	1.00	4.41	N.S.
3	C (Whey Solids)	2	5.3212	2.6606	6	1.03	19.00	N.S.
4	AB	2[c]	0.3082	0.1541	-	--	--	--
5	AC	4	15.5379	3.8845	7	6.05	6.39	N.S.
6	BC	2	5.1431	2.5716	8	7.01	3.55	Significant
7	ABC	4	2.5692	0.6423	8	1.75	2.93	N.S.
8	Error (Residual)	18	6.5892	0.3666				
9	Pooled Residual Error	20	6.8974	0.3449				

[a] See Table 7 for original data and levels used.

[b] F_o = Observed value; F_t = value from probability table.

[c] The AB interaction was found to have an F_o value less than 1.0, this indicates that it is an estimate of residual variance. Because of this it was pooled with the error term (residual variance) to obtain greater precision for testing (A) effects.

Table 7. Yield of Vitamin B_{12} in µgm/ml by Various Levels of Whey Solids and Yeast Extract.

Treatment		Replicate 1		Replicate 2		Average of all Det'ns.
Whey	Yeast Extract	Sample 1	Sample 2	Sample 1	Sample 2	
6%	0.5%	6.75	7.25	8.37	8.00	7.59
6%	1.0%	6.50	5.37	7.62	7.50	6.75
6%	1.5%	6.12	5.75	6.00	6.50	6.09
8%	0.5%	7.87	7.25	7.00	6.37	7.12
8%	1.0%	8.72	7.37	7.62	7.12	7.71
8%	1.5%	9.25	8.25	8.37	7.37	8.31
10%	0.5%	7.00	6.37	5.87	6.00	6.31
10%	1.0%	7.00	5.87	6.62	6.00	6.38
10%	1.5%	8.12	7.50	10.00	8.00	8.43

used. Within the 10% level of whey, the greatest amount of lactose remained with the 0.5% level of yeast extract (2.34%) and the lowest amount with the 1.5% level of yeast extract (1.48%). This seems to indicate a somewhat better rate of lactose utilization at the highest level of yeast extract as compared to the lowest level. This would seem to also indicate that a higher level of yeast extract is more adequate to supply growth factors necessary to support growth in the presence of a greater amount of fermentable material. The samples used to determine lactose levels were pooled from both replicates and

therefore represent a composite sample. The data are presented in Table 8.

Table 8. Percent Lactose Remaining at 168 Hours After Inocculation for the Various Treatments.

Treatment		
Whey	Yeast Extract	Percent Lactose
6%	0.5%	0.07
6%	1.0%	0.02
6%	1.5%	0.13
8%	0.5%	0.39
8%	1.0%	0.52
8%	1.5%	0.80
10%	0.5%	2.34
10%	1.0%	1.84
10%	1.5%	1.48

Determination of Optimum Levels of Cobalt Precursor and Aeration. In this experiment the effects of cobalt, 5,6-dimethylbenzimidazole precursor and aeration were studied. The levels of cobalt used were 5 ppm, 15 ppm and 25 ppm, the levels of precursor used were zero, 10 ppm and 15 ppm and the levels of aeration were zero, 215 ml of air/liter/minute and 1000 ml of air/liter/minute. Ten percent whey and 1.5% yeast extract were used and held constant in this experiment. The effect of aeration was the most striking. The

greatest difference was observed between no aeration and aeration. There appeared to be no difference between levels of aeration. The yields obtained with a low rate of aeration were very similar to the yield obtained with a high rate of aeration.

The 5,6-dimethylbenzimidazole precursor had a marked effect on the production of vitamin both in the presence and in the absence of aeration. In the absence of aeration the presence of precursor increased the yield of vitamin B_{12}. The zero aeration and zero precursor treatment produced only a small amount of vitamin B_{12}. There did not appear to be any advantage to a level of precursor higher than 10 ppm. The 15 ppm level of precursor did not appear to stimulate yields over that obtained at the 10 ppm level to any great extent and in some cases a slight decrease in yield was noted, however, the differences are within the limits of experimental error. In the presence of aeration the presence of precursor exerted a definite negative effect upon the levels of vitamin B_{12} obtained. Lower yields of vitamin B_{12} were obtained in the presence of both aeration and precursor, than were obtained with aeration alone. However, the yields of vitamin B_{12} obtained from the precursor and aeration treatment were similar, to slightly higher than those obtained with precursor alone. This would seem to indicate a possible interaction between aeration and precursor, and the statistical analysis, to be given later bears this out.

The effects of precursor and aeration observed in this experiment are similar to those observed by Speedie and Hull (1960). During the early stages of the fermentation the organism is forming the so called factor B which is converted to vitamin B_{12} by either the presence of the precursor under anaerobic conditions or by contacting the medium with air in the absence of precursor. The conditions relative to the effect of aeration and presence of precursor described by Speedie and Hull (1960) and others (Mervyn and Smith, 1964) seem to be equally true using whey and yeast extract as a production medium as opposed to the somewhat more defined media as used by Speedie and Hull.

While the third variable in this experiment, cobalt, is necessary for the production of vitamin B_{12}, little increased effect was observed by increasing the levels of cobalt beyond 5 ppm. Within each treatment studied, the higher levels of cobalt (15 ppm and 25 ppm) did not show any marked increase of vitamin over the lower 5 ppn level. This again is in accord with the results of Speedie and Hull who obtained good levels of vitamin B_{12} using 5 ppm of cobalt in a glucose and corn steep liquor medium. In a separate study on a smaller scale, a cobalt level of 2.5 ppm was studied using 10% whey 1.5% yeast extract, 215 ml of air/liter/minute the last one-half of the fermentation, and no precursor. The levels of vitamin B_{12} obtained in this study were comparable with those obtained using 5 ppm of cobalt. This would seem to indicate that 5 ppm of cobalt is quite adequate and probably even slightly in excess of what is required for the formation

of vitamin B_{12} by this organism. Since it is not thought that the 5 ppm level of cobalt exerts any toxic effect on the organism, it would seem logical to use a level of 5 ppm to insure adequate levels of the element. Since the relative cost of cobalt is not high when compared to the product being formed, this is not an economic factor.

The yields of vitamin B_{12} by the various treatments are presented in Table 10. These results are also shown graphically in Figures VI and VII.

An analysis of variance was performed on the data. It was found that aeration was highly significant. Interaction was observed between aeration and precursor, this figure also being significant. None of the other variables had significant effects, including precursor, cobalt and replications. There were no significant two factor interactions observed other than the aeration and precursor interaction. There were no significant three factor or four factor interactions observed. The error term or difference between subsamples was small. The analysis of variance is given in Table 9.

In the second replicate, the second subsample of the 1000 ml of air/liter/minute, zero precursor and 15 ppm cobalt treatment seems abnormally large when compared to the other values. This may well be experimental error within the assay technique. This figure did not adversely affect the statistical analysis, since the error term remained small, and because of this it was not considered to be a serious

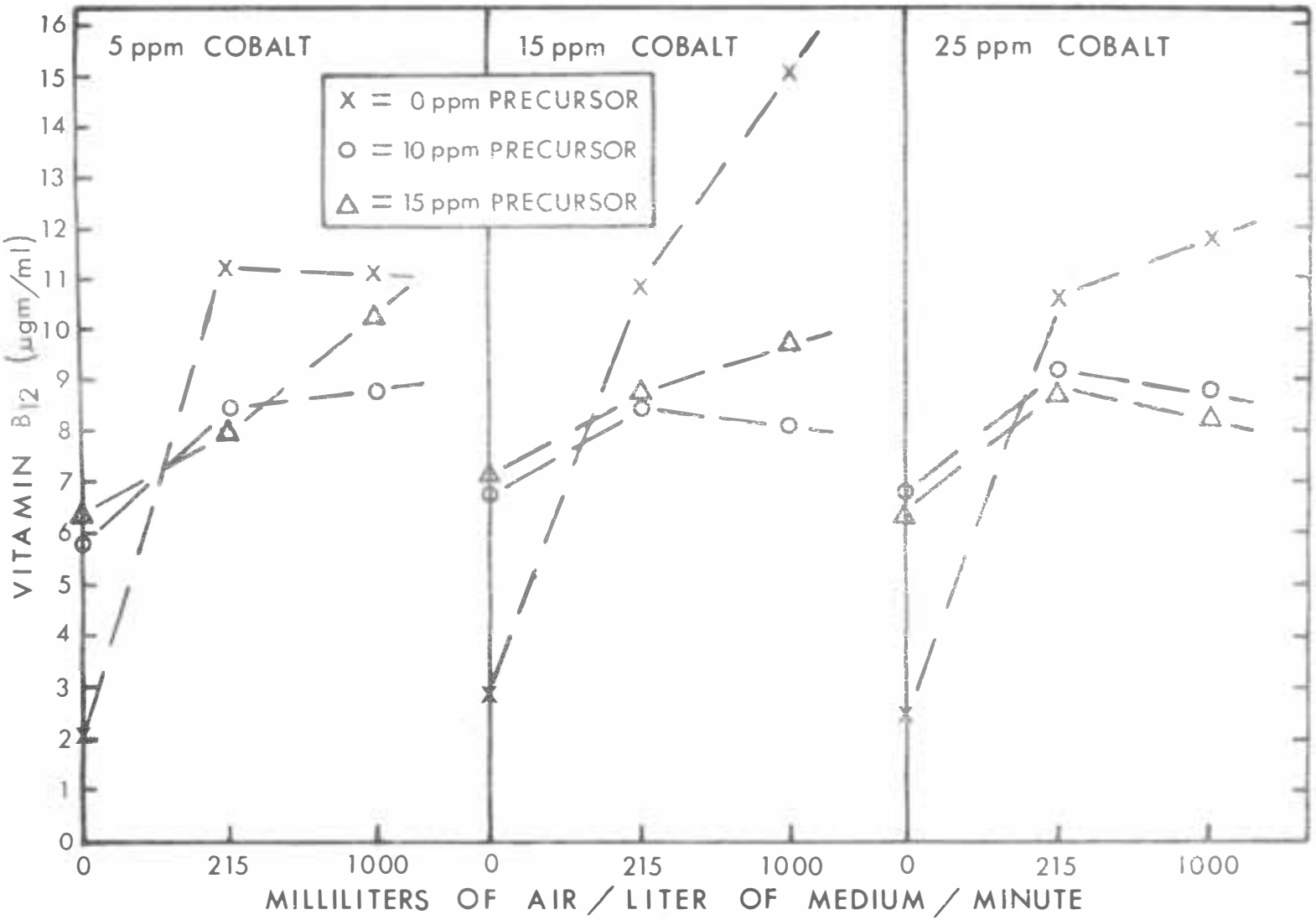

Figure VI. Effect of aeration on Vitamin B_{12} production at three levels of 5,6 - dimethylbenzimidazole precursor and three levels of cobalt.

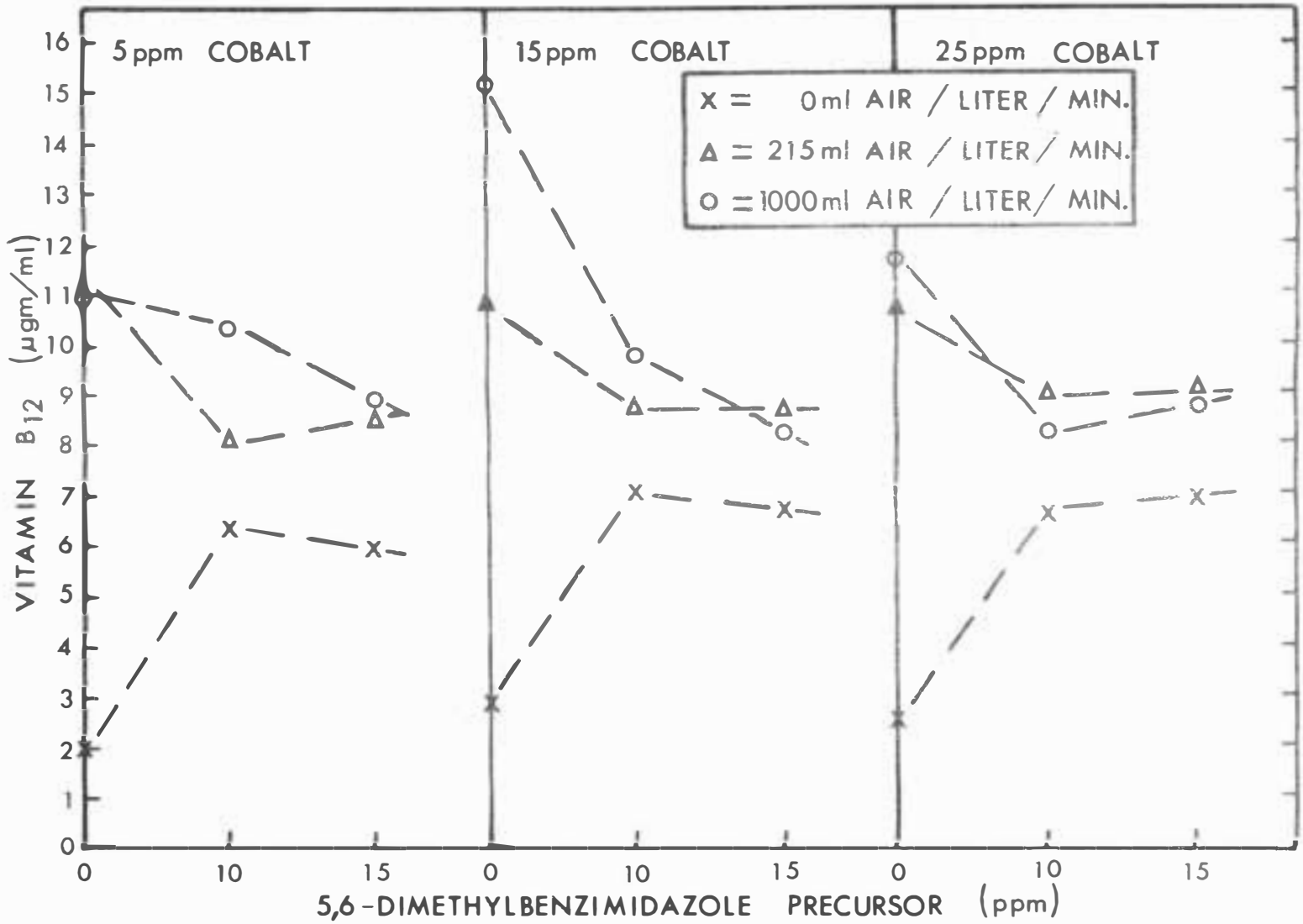

Figure VII. Effect of 5,6 - dimethylbenzimidazole on Vitamin B_{12} production at three levels of aeration and three levels of cobalt.

Table 9. Analysis of Variance of Vitamin B_{12} Production by *Propionibacterium Shermanii* for Three Levels of Cobalt, Three Levels of 5,6-Dimethylbenzimidazole Precursor and Three Levels of Aeration[a] Using 10% Whey and 1.5% Yeast Extract Medium.

Line No.	Effect	Degrees Freedom	Sum of Squares	Mean Squares	Error Testing Line	F_o[b]	F_t[b]	Significance
1	Total	107	1095.0021	---	---	---	---	---
2	A (Air)	2	514.3703	257.1852	6	45.38	9.55	Significant
3	B (Replications)	1	7.5577	7.5577	18	2.55	3.96	N.S.
4	C (Precursor)	2	10.1502	5.0751	18	1.71	3.11	N.S.
5	D (Cobalt)	2	6.3783	3.1892	18	1.08	3.11	N.S.
6	AB	3	16.9994	5.6665	18	1.91	2.72	N.S.
7	AC	4	269.0221	67.2555	18	22.69	2.48	Significant
8	AD	4	11.0539	2.7635	18	1	2.48	N.S.
9	BC	3	2.1238	0.7079	18	1	2.72	N.S.
10	BD	3	10.4766	3.4922	18	1.18	2.72	N.S.
11	CD	4	12.6821	3.1705	18	1.07	2.48	N.S.
12	ABC	4[c]	2.0148	0.5037	---	---	---	---
13	BCD	4	0.9811	0.2453	---	---	---	---
14	ABD	4	12.6410	3.1602	---	---	---	---
15	ACD	8	23.0553	2.8819	---	---	---	---
16	ABCD	8	16.8311	2.1039	---	---	---	---
17	Error (Residual)	51	178.6644	3.5032	---	---		
18	Lines 12 through 17 pooled	79	234.1877	2.9644	---	---		

[a] See Table 10 for original data and levels used.

[b] F_o = observed value; F_t = value from probability table.

[c] All three factor and four factor interactions had F_o values which were less than 1.0, this indicates that they are all estimates of residual variance. Because of this, these factors were pooled with the error term (residual variance) to obtain greater degrees of freedom and precision in testing remaining effects.

Table 10. Yields of Vitamin B_{12} (µgm/ml) Obtained Using Various Levels of Cobalt, Precursor and Aeration with 10% Whey and 1.5% Yeast Extract.

Treatment			Replicate 1		Replicate 2		Average Value
Air*	Precursor ppm	Cobalt ppm	Sample 1	Sample 2	Sample 1	Sample 2	For The Treatment
0	0	5	2.92	2.73	1.02	1.65	2.08
0	10	5	6.81	8.02	5.75	4.94	6.35
0	15	5	5.83	6.62	6.42	4.94	5.95
0	0	15	3.60	3.53	1.73	2.80	2.91
0	10	15	8.54	7.32	7.08	5.48	7.10
0	15	15	8.04	6.94	6.18	5.55	6.68
0	0	25	3.78	2.97	1.87	1.73	2.59
0	10	25	8.22	7.42	6.07	4.45	6.54
0	15	25	8.83	6.47	6.82	5.11	6.81
Low	0	5	12.52	10.90	12.57	8.83	11.20
Low	10	5	8.83	9.10	7.83	6.61	8.09
Low	15	5	8.37	9.58	8.64	7.42	8.50
Low	0	15	10.97	13.36	9.10	10.02	10.86
Low	10	15	9.36	7.76	8.83	8.69	8.66
Low	15	15	9.62	7.28	9.53	7.85	8.57
Low	0	25	9.63	9.36	10.87	12.92	10.64
Low	10	25	8.69	8.56	10.28	8.18	8.93
Low	15	25	10.32	7.14	9.99	8.77	9.05
High	0	5	14.21	10.20	11.64	8.46	11.13
High	10	5	12.04	9.22	10.70	9.22	10.30
High	15	5	9.45	9.58	7.42	8.91	8.84
High	0	15	12.69	13.63	10.29	23.54	15.04
High	10	15	9.49	10.30	8.69	10.43	9.73
High	15	15	9.31	6.86	8.12	8.73	8.25
High	0	25	11.37	10.44	10.02	14.58	11.60
High	10	25	8.95	7.62	7.28	9.14	8.25
High	15	25	9.62	6.36	7.83	11.20	8.75

*Low air, 215 ml/liter/minute; high air, 1,000 ml/liter/minute

defect in the data. However, upon graphic illustration of the data the effects of this sample are more visible. It is believed that the points which include the effect of this sample in Figures VI and VII

are not completely accurate, and that less consideration should be given to these points when considering the graphs. Since the variation in this one sample did not affect the statistical analysis and because of limited time, the sample was not rechecked. Also since three other determinations had been done within this treatment which gave lower readings and since all three agreed well, it follows that the large variation observed in this sample is very probably a false reading.

Growth Response of the Organism to the Conditions Studied. A comparison of growth rate, vitamin B_{12} formation, lactose utilization and pH changes by the organism in whey is shown in Figure VIII. The growth studies were conducted using 10% whey, 1.5% yeast extract, 5 ppm cobalt, 215 ml air/liter/minute the last one half of the fermentation and no precursor. From the data it can readily be seen that the organism approaches its maximum growth before appreciable quantities of vitamin B_{12} are produced. This is consistent with the literature concerning vitamin B_{12} production in other substrates (Speedie and Hull, 1960; Mervyn and Smith, 1964). The growth of the organism and production of the vitamin do not seem to vary, due to the whey substrate, from the patterns established using other types of substrates. The lactose of the whey appears to be readily available to the organism for growth, since great decreases in lactose occurred during the logrithmic growth phase of the organism.

From the results of the growth studies it can be seen that the growth pattern and the pattern of vitamin formation are normal with

respect to existing knowledge. From the data it would seem that the whey and yeast extract are capable of supplying the necessary materials for growth and vitamin formation by the organism. However, it cannot be said that these materials are necessarily present at optimum levels, but rather that the substrate supports the desired activity of the organism. These data are presented in Table 11.

Vitamin B_{12} and Feed Analysis of Dried Culture Solids Concentrate. Three separate determinations of vitamin B_{12} activity of a dried portion of the fermentation material were made. At the same time three separate determinations of the vitamin B_{12} activity of dried unfermented whey were made. The results are given in Table 12. From the data in Table 12 it can readily be seen that a very large increase in vitamin B_{12} occurs in the substrate during the course of a fermentation when compared to dried unfermented whey on a moisture free basis. The difference in levels of vitamin B_{12} between fermented and unfermented whey results from the activity of the organism.

A feed analysis was also performed on the material. The results of this analysis are compared with figures for dried whey in Table 13. The figures for analysis for dried whey were taken from Composition of Concentrate By-Product Feeding Stuffs, June 1956. As can be seen from Table 13, there was a notable increase of crude protein and a notable decrease of nitrogen free extract of the fermentation material over the unfermented dried whey. There was also a rather large increase of crude fiber. One possible explanation of the

Table 11. Vitamin B_{12} Formation, Growth, Lactose Utilization and pH Changes in Whey, at Increasing Culture Times by Propionibacterium Shermanii.

Time (Hours)	Vitamin B_{12} (µgm/ml)	Cell Numbers (X 10^{11})	Lactose Percent	pH	Adjusted pH
0	0.01	0.09	7.43	6.20	--
6	0.03	--	7.07	--	--
18	0.19	0.32	6.91	--	--
24	--	--	--	5.25	7.25
30	0.10	--	6.71	--	--
48	0.34	4.1	5.37	5.00	6.85
63	0.52	8.3	4.19	--	--
72	0.87	8.3	2.90	5.10	6.75
98	1.87	12.0	2.63	5.50	6.95
108	3.12	11.0	2.36	--	--
120	--	11.0	--	5.55	7.60
136	3.75	--	1.34	--	--
144	--	--	--	5.70	7.15
168	15.87	--	1.25	5.80	7.05
192	17.37	--	1.35	6.20	6.85
216	20.87	--	1.19	6.75	--

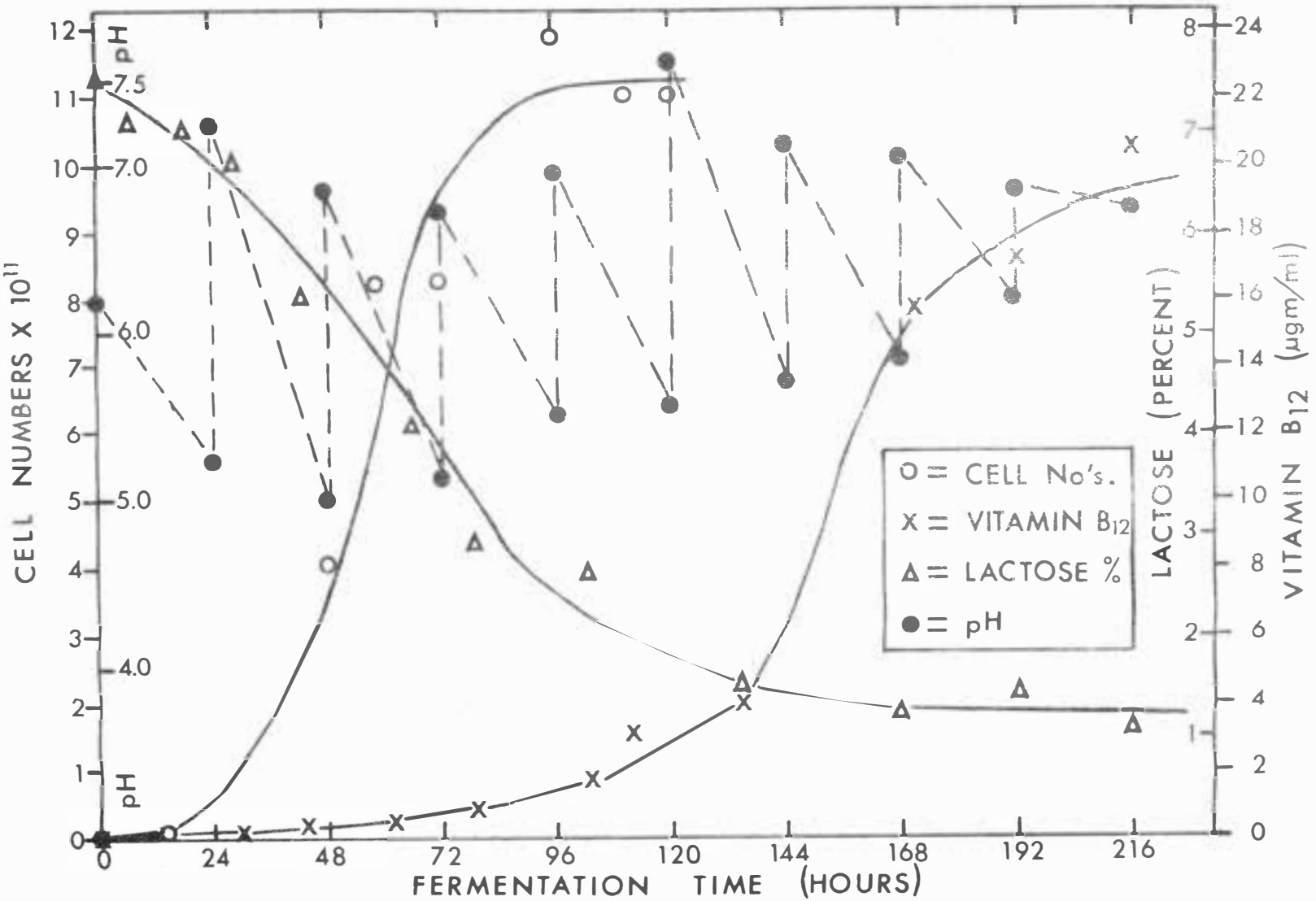

Figure VIII. The relationships of growth, lactose utilization, pH and Vitamin B_{12} formation in whey by Propionibacterium shermanii.

Table 12. Vitamin B_{12} Levels of Dried Culture Solids and Dried Unfermented Whey.

Sample	Vitamin B_{12} (µgm/gm) First Det'n	Second Det'n	Third Det'n	Average All Det'ns	Vitamin B_{12} MFB*
**Unfermented dried whey	0.013	0.006	0.054	.024	0.025
Dried fermentation material	330	435	397	387	399

* MFB - moisture free basis. (Moisture content after drying was 3.0%)

** To determine vitamin B_{12} content of dried whey MFB the dry matter percent was taken from the publication Composition of Concentrate By-Product Feeding Stuffs, June 1956. National Academy of Sciences National Research Council publication 449.

Table 13. Feed Analysis of Dried Culture Solids and of Dried Unfermented Whey.

Analysis*	Dried Unfermented Whey	Dried Fermentation Material
Dry Matter	94.00%	97.00%
Ether Extract	0.50%	0.46%
Crude Fiber	0.30%	7.19%
Crude Protein	13.10%	63.67%
Ash	8.30%	4.99%
Nitrogen Free Extract	72.50%	24.77%

*All figures except percent dry matter are moisture free basis.

increased crude fiber might be that it is due to the presence of charred materials. The material darkened considerably on drying. It was assumed that the dark color was due to charring, and that the charring was due to heating either when drying or when autoclaving.

On the basis of this work alone, it is difficult, if not impossible, to make any meaningful statement relative to the economics of a commercial fermentation using whey as a substrate. However, the results based on a dry weight basis are encouraging and compare favorably with levels of vitamin B_{12} in commercial pre-mix feed concentrates.

The deciding factor concerning a production process for vitamin B_{12} using whey as a substrate will be economics. The work reported here indicates that whey is a suitable substrate for vitamin B_{12} production by Propionibacterium shermanii. However, the fact remains that if whey is to be used as a substrate for commercial vitamin B_{12} production processes it will have to compete with other types of substrates, many of which can be obtained quite cheaply. In this respect, an inherent disadvantage of whey is the fact that much of the surplus whey is produced by small, widely scattered cheese plants, whose individual production is not large enough to support a profitable production process for vitamin B_{12}. If such a process were to utilize whey as a substrate, very probably the whey would have to be transported to a central location where large quantities could be assembled and processed. The transportation would increase the cost of the

substrate, since whey is high in water (93.1%) and low in solids (6.9%). However, if the whey could be concentrated to a high solids level at the point of production and then transported, the costs would be reduced.

Since vitamin B_{12} has been observed in sewage sludge, (Hoover, et al., 1951; Hoover, et al., 1952) another possibility may be to decrease production costs by combining whey and sewage for vitamin B_{12} production for use in animal feed supplements. The sewage would supply nitrogen while the whey would supply lactose which is readily available to sewage organisms for growth. A possible combination of whey and other types of wastes such as sewage or farm animal wastes may well result in processes for utilization of these wastes for animal feed purposes.

SUMMARY

The suitability of cheese whey as a substrate for vitamin B_{12} production by Propionibacterium shermanii was studied. It was found that the patterns of growth and vitamin formation by the organism in whey were similar to the patterns established in other substrates. The vitamin formation was observed during the latter part of the fermentation after the organism approached maximum growth, or at about 80% to 100% of the total growth. Lactose utilization by the organism corresponded to the logarithmic growth phase of the organism as expected.

Within the limits of the study it was found that levels of 10% whey and 1.5% yeast extract gave the best yields. A 5 ppm level of cobalt was found to be adequate, since higher levels of cobalt did not appreciably stimulate yields of the vitamin. The precursor 5,6-dimethylbenzimidazole precursor was found to be adequate at 10 ppm in the absence of aeration. In the presence of aeration, a zero level of precursor was found to be most desirable.

A dried portion of the fermentation material was found to contain a high amount of vitamin B_{12} activity, as well as considerable amounts of protein. The vitamin B_{12} content on a dry weight basis was found to be about 400 µgm/gm. The material contained about 64% crude protein on a dry weight basis.

CONCLUSIONS

On the basis of the data presented in the foregoing discussion it is concluded that whey is a suitable substrate for the production of vitamin B_{12} by Propionibacterium shermanii. The organism grows well in whey, and produces vitamin B_{12}. The pattern of growth and vitamin production are the same in whey as in other substrates.

The yields of vitamin B_{12} on a per milliliter basis as compared to values in the literature are intermediate when the fermentation is carried out under the conditions of this study. It is believed that with higher yielding strains of the organism and possibly more nearly optimal culture conditions, higher yields would be possible.

Further work on this subject is warranted and is needed. The information obtained in this study is not enough to make valid decisions concerning commercial production of vitamin B_{12} using whey as a substrate. Additional studies should include pilot plant studies.

Further work should also be done to determine more accurately the optimum conditions for the production of vitamin B_{12} in whey by P. shermanii. Additional levels of whey solids should be studied. Possibly a somewhat higher level of whey solids would be desirable. Levels of 12% and 14% whey solids when used with the proper amount of yeast products may well give higher yields. It is believed from the

results of this study that the proper ratio of whey solids to yeast extract is critical. If higher levels of whey were studied the proper level of yeast products would have to be established to obtain highest yields.

It is also thought that an additional source of nitrogen other than whey nitrogen or ammonia nitrogen may be desirable. Since many commercial media for the production of vitamin B_{12} employ corn steep liquor, it is believed that the addition of corn steep liquor to the whey may stimulate yields. However, it is thought that the highest yields of vitamin B_{12} would probably result from a slightly higher level of whey solids combined with a more optimal adjustment of the ratio of whey solids to yeast extract.

Within the conditions of this experiment, the cobalt level is adequate at 5 ppm. In the absence of aeration the 5,6-dimethylbenzimidazole precursor is required, and 10 ppm is an adequate level for this material. However, in the presence of a low rate of aeration the last one-half of the fermentation the precursor is not required. More work should be done to establish the desirability of using a very low level of precursor, (2.5 to 5 ppm) in conjunction with aeration to insure the animal active, 5,6-dimethylbenzimidazole form of vitamin B_{12}. The reaction of the organism to cobalt levels and the interrelationships of aeration and precursor are the same in whey as in other types of substrates.

On the basis of the data presented, it is believed that the production of vitamin B_{12} in whey by P. shermanii is subject to the same type of culture conditions as in other substrates. The whey substrate causes no variation in the behavior of the organism with respect to growth and vitamin formation as established in other substrates.

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